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Transmission Networks and Population Turnover of Echovirus 30[†]

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Globally, echovirus 30 (E30) is one of the most frequently identified enteroviruses and a major cause of meningitis. Despite its wide distribution, little is known about its transmission networks or the dynamics of its recombination and geographical spread. To address this, we have conducted an extensive molecular epidemiology and evolutionary study of E30 isolates collected over 8 years from a geographically wide sample base (11 European countries, Asia, and Australia). 3Dpol sequences fell into several distinct phylogenetic groups, interspersed with other species B serotypes, enabling E30 isolates to be classified into 38 recombinant forms (RFs). Substitutions in VP1 and 3Dpol regions occurred predominantly at synonymous sites (ratio of nonsynonymous to synonymous substitutions, 0.05) with VP1 showing a rapid substitution rate of 8.3×10^{-3} substitutions per site per year. Recombination frequency was tightly correlated with VP1 divergence; viruses differing by evolutionary distances of >0.1 (or 6 years divergent evolution) almost invariably ($>97\%$) had different 3Dpol groups. Frequencies of shared 3Dpol groups additionally correlated with geographical distances, with Europe and South Asia showing turnover of entirely distinct virus populations. Population turnover of E30 was characterized by repeated cycles of emergence, dominance, and disappearance of individual RFs over periods of 3 to 5 years, although the existence and nature of evolutionary selection underlying these population replacements remain unclear. The occurrence of frequent “sporadic” recombinants embedded within VP1 groupings of other RFs and the much greater number of 3Dpol groups than separately identifiable VP1 lineages suggest frequent recombination with an external diverse reservoir of non-E30 viruses.

The genus *Enterovirus* in the family *Picornaviridae* is a group of nonenveloped RNA viruses that cause a wide range of diseases in humans and other mammals. Enteroviruses contain a positive-sense RNA genome of approximately 7,500 nucleotides encoding a polyprotein that after cleavage yields structural (capsid proteins VP1 to VP4) and nonstructural (2A to 3D) proteins. Primary infection with an enterovirus leads to viral replication in the tissue around the gastrointestinal tract, followed by a transient viremia and sometimes migration into other tissues (6, 44). Although infection in immunocompetent individuals is often asymptomatic or causes mild febrile illness, enteroviruses are a common etiological agent in aseptic meningitis, encephalitis, and paralysis in individuals of all ages, with persistent and/or widely disseminated systemic infection in immunosuppressed individuals and neonates (12, 19, 23).

Enteroviruses were originally classified as polioviruses, coxsackie virus type A or B viruses, or echoviruses (enteric cytopathic human orphan viruses), depending upon the infectious

properties of the virus such as pathogenicity in mice (reviewed in reference 22). From the 1960s onwards, enteroviruses within these groups were further differentiated into serotypes originally by using panels of specific neutralizing antisera and, more recently, by sequence comparisons of structural gene regions such as VP1 (9, 34, 38, 43). There are currently over 100 recognized human enterovirus serotypes that fall into four main species (designated A to D) using phylogenetic analysis (54). The *Enterovirus* genus additionally contains several other species infecting primates, cattle, and pigs and has recently been expanded to include the genetically related human rhinovirus A and B (54).

The species B serotype, echovirus 30 (E30), is a major cause of meningitis in both children and adults. Among the many serotypes associated with this disease presentation, E30 is generally the most commonly isolated in Europe (8, 31, 49), the United States (10, 37), Asia (1, 60), and South America (33). E30 infections typically occur as a series of outbreaks every 3 to 5 years, frequently over large geographical areas. For example, high frequencies of E30 detection in meningitis cases and surveillance programs were reported for 2000 to 2001 throughout Europe, including Denmark (58), Belgium (57), Cyprus (45), Germany (46), and France (3, 5), and again in 2005 to 2006 (8). Similarly, in the United States, long-term surveillance

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TABLE 1. Sources of survey specimens

Country	No. of isolates	Date(s) of isolation
Australia	18	2000
Azerbaijan	2	1999
Spain	69	2000–2006
Finland	26	2000–2007
UK	60	2000–2007
Georgia	4	1999–2000
Croatia	2	2005
Ireland	24	1998–2006
Iceland	1	2007
Lithuania	3	2004
Moldova	1	2002
Malaysia	23	2001–2004
Netherlands	18	1997–2007
Russia	11	1999–2003
Slovenia	3	2001
Ukraine	5	1999–2000
Total ^a	325	1998–2007

^a The total includes 55 previously published isolates from 1958 to 2003.

by the Centers for Disease Control and Prevention revealed peaks of E30 isolation in 1981, 1991 to 1993, 1997, and 2003 (10, 37). The underlying basis for this periodicity in E30 infections and the possible association of different genetic variants of E30 with outbreaks are currently poorly understood.

At any one time point, a range of different species B enterovirus serotypes circulate in human populations. The evolution of enteroviruses occurs through genetic drift and, over much longer periods, antigenic diversification in the structural gene region encoding the virus capsid (7, 14, 25, 30, 51, 55); it may also occur by recombination between the capsid and nonstructural coding parts of the genome and the 5' untranslated region (2, 13, 16, 20, 26, 28, 29, 35, 39, 41, 47, 48, 53). To date, almost all documented examples of recombination have been limited to members of the same species (e.g., between species B serotypes), with the exception of the 5' untranslated region, where only a single genetic group can be identified within human species A and B and a second with species C and D (48).

In this study, we have carried out an extensive investigation of VP1 sequence divergence and recombination through sequencing the 3Dpol region of E30 isolates and samples collected from several European countries, Southeast Asia, and Australia over a combined 8-year observation period. Using this geographically diverse sample collection, our aims were to document the time span and geographical extent of different E30 variants as they emerged and spread during the observation period. The identification of individual recombinants of E30 provides the means to document in detail the dynamics of E30 population turnover, geographical ranges of enterovirus transmission networks, and, ultimately, the relationship between the emergence of new variants of E30 and longer-term changes in disease associations and pathogenicity.

MATERIALS AND METHODS

Samples. E30 isolates for the study were collected from several globally distributed sites between the years 1998 to 2007 (Table 1). Isolates were named using the following convention: two-letter country code plus the isolate number/two-letter city or region abbreviation/3Dpol clade/last two digits of the year of

collection (e.g., ES10/Ma/C/00 for sample 10 referred from Madrid, Spain [ES], isolated in 2000, and belonging to the 3Dpol clade C). Sequences obtained in the current study were supplemented with those of published sequences of E30 where both VP1 and 3Dpol sequences overlapped with those we obtained. These included the following types of sequences: published complete genome sequences of the prototype Bastianni isolate (accession no. AF311938); and isolates from The Netherlands (DQ534205), China (DQ246620 and AY948442), and Taiwan (EF066391 and EF066392); French isolates sequenced in the VP1 and 3Dpol regions (32); and isolates from Russia and the New Independent States with previously published sequences for the VP1 region (27) which were sequenced in the 3Dpol analysis in the current study.

Amplification of VP1 and 3Dpol regions. Nested reverse transcription-PCRs (RT-PCRs) were performed using species B-specific enterovirus primers designed to amplify a 395-bp region of the VP1 gene or a 549-bp region of the 3Dpol gene (53). A combined RT and first-round PCR was accomplished utilizing the Superscript III One-step RT-PCR system with platinum *Taq* DNA polymerase (Invitrogen) with component concentrations as outlined in the product information. Cycling conditions used were as follows: 1 cycle of 43°C for 1 h; 20 cycles of 53°C and 55°C for 1 min each; 1 cycle of 70°C for 15 min; 1 cycle of 94°C for 2 min; 40 cycles of 94°C for 30 s, 50°C for 30 s, and 68°C for 90 s; and a final extension of 68°C for 5 min. The second-round PCR, using GoTaq DNA polymerase (Promega), was performed as described in the product information but with the following final reagent concentrations: deoxynucleoside triphosphates, 0.03 mM (each); primers, 0.5 µM (each); and polymerase, 0.4U/µl. Cycling conditions were 94°C for 18 s, 50°C for 21 s, and 72°C for 90 s for 30 cycles, with a final extension of 72°C for 5 min. For the 3Dpol region, primers with the following sequences were used: GGY GGY ACH CCC CAN AAR AGA ATG CT (outer sense), TCR TCY TTB ACR TAD GTY ACC ATT GG (outer antisense), AAR AGA ATG CTY ATG TAY AAY TTY CC (inner sense), and ARD CCR TAY TTR TCC ATR CAY TCY TT (inner antisense). For VP1 amplification, the following primers were used: CCA TTC ATG AGY GTT GGY AAC GCA TA (outer sense), TGY ACC TCM AYG AGC CCR GGC CCT TC (outer antisense), TAY TGY AAC TTY TAT GAT GGA TGG TC (inner sense), and ARG TCT CTA TTG TAR TCC TYC CAC AC (inner antisense). For the samples from Russia and the New Independent States, amplification of the 3Dpol region was achieved using cDNA previously generated (27) in a nested PCR using the primer pairs shown above but using the GoTaq polymerase method for both amplification rounds.

Nucleotide sequencing. Amplified DNA from RT-PCR-positive pools was directly sequenced using BigDye (ABI) with the inner sense or antisense primer used for amplification, followed by annotation and alignment of E30 nucleotide sequences using the Simmonic sequence package, version 1.6 (52; <http://www.virus-evolution.org>).

Phylogenetic analysis. Phylogenetic trees were constructed by neighbor joining from 1,000 samplings of maximum-composite-likelihood (MCL) distances using the MEGA4.0 software package (56) with pairwise deletion for missing data. Regression analysis used MCL distances (calculated by MEGA [56] with a gamma distribution value, α , of 0.8) between each sequence and the early isolate, 14916net87. A Markov chain Monte Carlo (MCMC) method implemented in the BEAST package, version 1.4 (17), was used to estimate the rate of sequence change (18). Dated sequence sets were run three times with chain lengths of 50 million under the SRD06 model of substitution (50) and assumptions of a strict clock and constant population size. All other parameters were optimized during the burn-in period. Output from BEAST was analyzed using the program TRACER (<http://beast.bio.ed.ac.uk/Tracer>).

Nucleotide accession numbers. Sequences obtained in this study have been deposited in the GenBank database under accession numbers FJ525954 to FJ526200 and FJ538597 to FJ538865.

RESULTS

Sequence diversity of E30. Isolates from referral centers (Table 1) were sequenced in the VP1 region between positions 3062 to 3334 (numbering based on the Bastianni prototype strain, accession no. AF311938). Comparison of VP1 sequences from previous studies allowed the main clades to be matched to previously assigned genotypes or lineages. The majority (318/325) of variants analyzed in the study fell into previously assigned E30 genotype II cluster of VP1 sequences, with most falling into the H lineage and smaller numbers in the

TABLE 2. Estimates of rates of sequence change of E30 by regression and BEAST analysis

Region	Divergence	<i>dN/dS</i>	Regression		MCMC (BEAST) ^b	
			<i>R</i>	<i>P</i> ^a	Substitution rate (10 ⁻³) ^c	MRCA
VP1	0.113	0.04	0.341	4.3 × 10⁻¹⁰	8.8 (7.4–1.04)	1977 (1968–1985)
3Dpol	0.276	0.05	0.107	0.06	ND	ND

^a Significant value is shown in boldface (*P* < 0.05).

^b The mean value is given with the HPD interval in parentheses. ND, not done.

^c Number of substitutions per year.

E, F, and G VP1 subgroups designated in previous studies (8, 42). These correspond to VP1 lineages classified as C0 to C4 (32) or 3a to 3d (49). The more divergent genotype I sequences not analyzed in the current study comprised four sequences from Malaysia (MY18, MY19, MY22, and MY23; collected in 2004), one from London, United Kingdom (GB60; 2006), and one from Finland (FI30; 2007), along with the published sequence Bastianni (United States; 1958).

VP1 sequences from the main E30 group in the sequenced region of VP1 showed approximately 9% nucleotide sequence divergence (mean pairwise *P* distance), with variability almost entirely restricted to synonymous sites (nonsynonymous to synonymous substitution [*dN/dS*] ratio based on uncorrected synonymous and nonsynonymous distances of 0.04). The evidence for neutral drift rather than positive (Darwinian selection) in the evolution of this region justifies measurement of its rate of sequence change to estimate the time of the most recent common ancestor (MRCA) of this subgroup of E30 isolates. There was a significant correlation between sequence distance from the earliest main group E30 isolate (14916net87) and distances of sequences from more recently collected isolates (317 sequences; *R* = 0.341; *P* = 4.3 × 10⁻¹⁰), providing evidence for measurable sequence drift of E30 over the observation period.

The same data set was analyzed by the Bayesian MCMC method (18). A relaxed (log normal) molecular clock fitted the data better than a strict clock (log likelihoods of -4,895 and -4,966, respectively; log₁₀ Bayes factor of 30.6). Using the relaxed clock, the nucleotide substitution rate was calculated as 8.8 × 10⁻³ (high-probability distribution [HPD] range, 7.4 × 10⁻³ to 1.04 × 10⁻³) substitutions per site per year (Table 2). MCMC predicted a date for the MRCA of the main group of E30 variants of 1977 (HPD range, 1968 to 1985) (Table 2). A similar analysis of a data set that included the older Bastianni prototype isolate (collected in 1958) produced similar substitution rates (8.5 × 10⁻³ [HPD range, 7.0 × 10⁻³ to 9.9 × 10⁻³] substitutions per site per year using a relaxed clock), but an earlier MRCA (1948 [HPD range, 1933 to 1958]), consistent with the earlier isolation date of the oldest collected sequence.

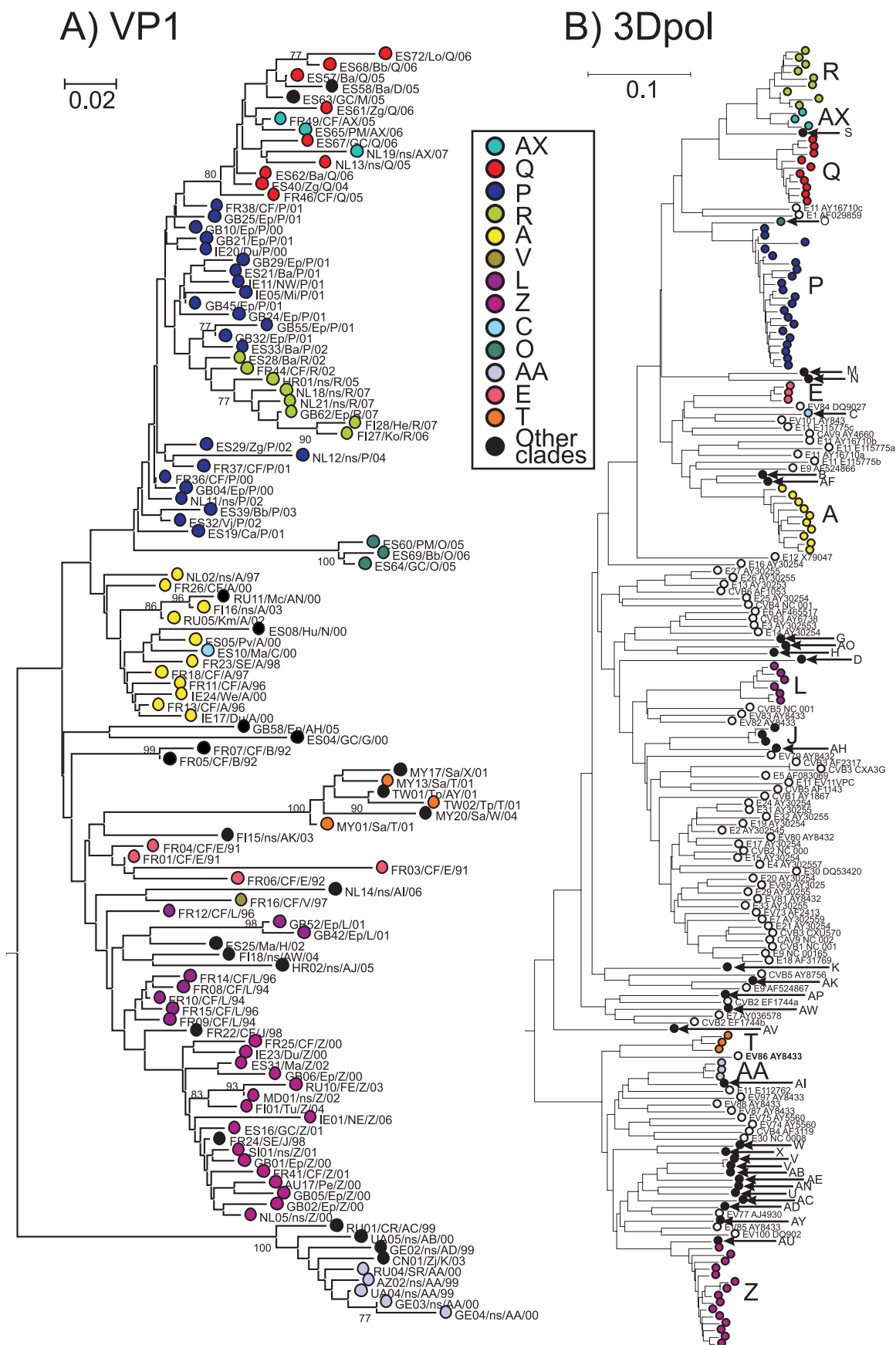
Variability in the 3Dpol region for the same subset of sequences was greater (Table 2) but with synonymous changes still accounting for most of the sequence divergence (*dN/dS* ratio of 0.05). However, unlike VP1, there was no significant association between sequence divergence from the early modern E30 isolate, 14916net87, and time (Table 2), consistent with a slower substitution rate or different underlying mechanisms of sequence change. Supporting the latter possibility, phylogenetic relationships between 3Dpol sequences in this region were distinct from those of VP1. First, 3Dpol sequences

formed a series of discrete clusters (Fig. 1B) rather than the continuum of progressive time-correlated drift observed in VP1. Second, 3Dpol sequences of E30 were not monophyletic; instead, sequences were interspersed with 3Dpol sequences of other serotypes within species B, with no evidence for any preferential grouping together of E30 isolates. None of the E30 3Dpol groups contained sequences from other serotypes.

The difference in tree structure is reflected by distinct distributions of pairwise distances in the two regions (Fig. 2); sequences within phylogenetic groups were invariably below 0.08 (mean MCL distance, 0.023), and with a few exceptions in the boundary zone, pairwise distances between groups were greater than this threshold (mean value, 0.320). We have used phylogenetic clustering and this distance threshold to classify E30-associated 3Dpol sequences into a total of 38 clades in the analysis presented in the rest of the paper.

Temporal and geographical correlates of recombination in E30. The identification of separate clusters of 3Dpol sequences provides the means to identify individual recombinant forms (RFs) of E30 (labeled after their assigned 3Dpol clade [Fig. 1B]), providing the means to follow the turnover of separate populations of variants of this serotype over time and distance. Clustering together in VP1 of sequences with the same 3Dpol group demonstrates at least short-term genetic linkage between the structural gene region and 3Dpol (Fig. 1A). Thus, with some exceptions analyzed below, sequences assigned as RF-A (Fig. 1A) formed an approximately monophyletic group in VP1, as did RF-R and RF-Z. There was a consistent relationship between VP1 divergence and frequency of pairwise comparisons between variants with different 3Dpol groups (Fig. 3A). This can be conceptualized as the length of time (and therefore sequence divergence) of an enterovirus population before recombination occurs to take one or other descendant virus in the pairwise comparison into another 3Dpol group.

The threshold distance where 50% of sequence comparisons were recombinant was approximately 0.05 (Fig. 3A). Using the substitution rate calculated above (Table 2), this corresponds to a period of 5.81 years (0.05/0.0086) of divergent evolution, or approximately 3 years from a common ancestor. Direct temporal comparisons were complicated by differences between E30 variants in their geographical separation (Fig. 3B). However, the subset of comparisons between variants from the same city revealed a time-related relationship with recombination that was consistent with the VP1 divergence data. For these, most sequence comparisons between variants collected in the same year showed the same 3Dpol group, while between 1 and 3 years, 76% were recombinant, and almost all comparisons (96%) between variants >3 years apart were recombinant.



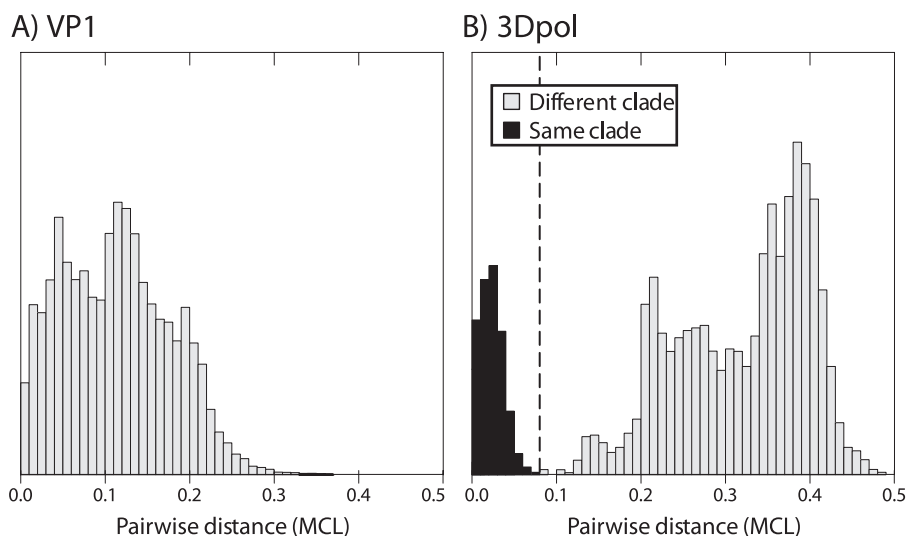


FIG. 2. Distributions of pairwise evolutionary distances between sequences from study subjects and published E30 sequences in the VP1 region (A) and 3Dpol (B). In the latter, the dotted line represents the threshold between intra- and interclade distances.

The relationship between recombination and geographical separation was separately analyzed. Removing the time element by comparing variants collected in the same year (Fig. 3B) indicated that variants collected in the same city or within 1,000 km (generally within Europe for this data set) were mostly nonrecombinant, in contrast to >80% for variants 1,000 to 3,000 km apart and >95% of those collected more than 3,000 km apart. The only exception were E30 variants collected in Australia; they showed the same 3Dpol sequences as those in Western Europe in their collection year (2000) (see next section). Combined, these data are consistent with relatively large although geographically restricted transmission networks for each E30 RF and with relatively rapid temporal turnover of variants within individual geographical zones.

Turnover of E30 in a single geographical area. Western Europe was the most intensively sampled region in the current study and provided the opportunity to investigate the turnover of individual RFs with time (Fig. 4). Turnover of E30 variants occurred throughout Western Europe approximately synchronously, as indicated by the low frequency of comparisons between different RFs in the same calendar year for sequences collected <1,000 km apart (40% compared with 22% for variants from the same city).

There were marked changes in frequencies of detection of individual RFs between years and evidence for rapid time-related turnover. For example, at the start of the observation period, RF-V and RF-A were the most prevalent, but in subsequent years, first RF-V then RF-A disappeared to be re-

placed with RF-P as the predominant strain in 2001. It was subsequently replaced by RF-Z (predominant in 2004), then RF-Q (2005 to 2006), and finally RF-R (2007). Each of the common RFs appeared to circulate in the European study subjects for 3 to 5 years, a period consistent with previous analyses of time-VP1 divergence-correlated recombination frequencies (Fig. 3).

We have insufficient sampling outside of Europe to clearly define the extent of the transmission networks within which this turnover of E30 occurred. However, on the boundaries of Europe, different RFs were observed; RF-AA (not found in Europe) was the most commonly detected RF in southern Russia, Georgia, and Azerbaijan in 2000, and RF-A was detected in southern Russia in 2002 despite its virtual disappearance from Europe by that time. Further from Europe, in Southeast Asia, E30 variants in Malaysia (this study), Taiwan (11), and China (60) were entirely distinct. RF-T accounted for 16 of 18 E30 variants found in Malaysia and Taiwan in 2001, to be replaced by RF-F and RF-W in 2003 and 2004. (None of these RFs was found in Europe.) Despite this evidence for the circulation of a different set of E30 variants in Southeast Asia, variants collected in 2000 from Perth, Australia, were RF-P and RF-Z, the two most common RFs in Europe in that year. What these findings imply for transmission routes of E30 and potentially other enteroviruses is discussed below.

Minor RF populations. Circulating populations of E30 are dominated by a series of major RFs that show time-related turnover. In Europe, just 10 of the 26 RFs observed (38%; RF-AX,

FIG. 1. Neighbor-joining tree of MCL-corrected pairwise distances of VP1 sequences (A) and 3Dpol sequences (B) from the study subjects. Because of the number of sequences (>300), only those showing greater than 1.0% or 1.5% divergence, respectively, from each other are shown. Dot colors indicate the 3Dpol clade of the displayed sequences (see text), not the H, E, F, and G VP1 subgroups assigned in previous analyses (8, 42). 3Dpol region sequences of study subjects were interspersed with sequences from other species B serotypes and have been included in panel B, labeled with the following abbreviations: E, echoviruses; CVA, coxsackie virus type A; CVB, coxsackie virus type B; EV, new enteroviruses. Accession numbers are given. Clades containing three or more members are colored. The scale bar depicts an evolutionary distance of 0.02. Bootstrap resampling was used to determine robustness of the groupings; values of $\geq 70\%$ are shown.

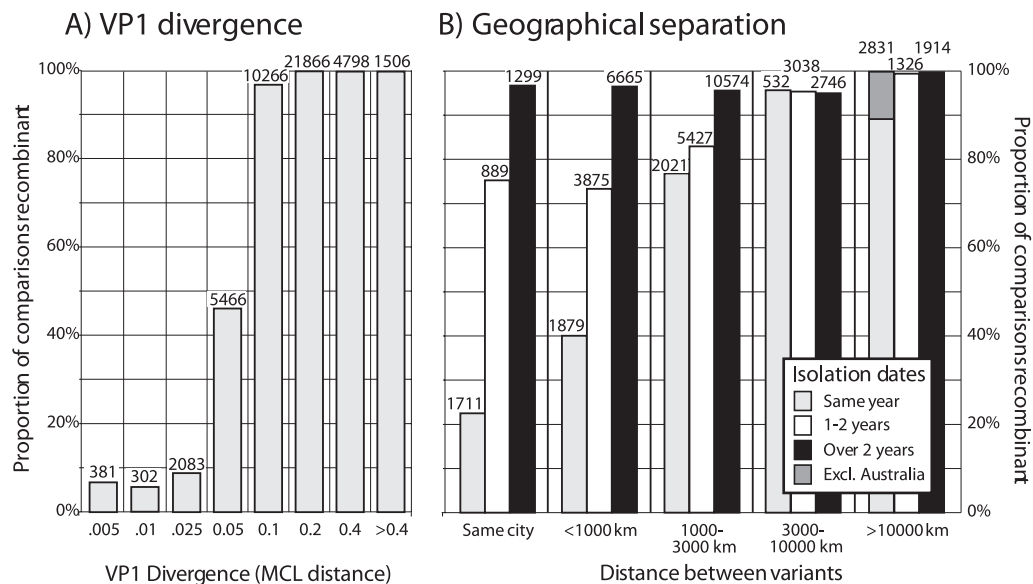


FIG. 3. Association between VP1 sequence divergence (x axis shows maximum distance in each category) (A) and geographical/temporal separation with recombination frequency (y axis) (B). This records the proportion of pairwise comparisons between sequences that show different 3Dpol clades in each category (total numbers of pairwise comparisons are shown above bars). In panel B, for comparison of sequences of variants >10,000 km apart, separate proportions have been calculated for data sets including (light gray) and excluding (dark gray) sequences from Australia.

-Q, -P, -R, -A, -V, -L, -Z, -J, and -O) accounted for 92% (222/240) of isolates characterized over this period, whereas 12 RFs were found only once. These 12 “sporadic” RFs frequently showed different patterns of recombination from those of the main RFs

(Fig. 5; Table 3), typically falling into VP1 lineages normally associated with a different 3Dpol group. For example, a well-defined, bootstrap-supported lineage in the VP1 region contained 17 RF-T isolates from Malaysia and Taiwan and a single, sporadic isolate (TW01/Tp/AY/01) with an AY 3Dpol group (Fig. 5). Similarly, a large lineage of European RF-Z isolates contained several sporadic RFs (RF-U, -S, -J, and -AV) interspersed with RF-Z variants. In total, we observed 12 sporadic RFs, each with a 3Dpol group unique to that isolate. A further two isolates clustered with RF-Z isolates in VP1, but 3Dpol sequences fell in the J clade, which included a third E30 isolate with an entirely different VP1 sequence. Collectively, these isolates break the previously observed association between VP1 divergence and linkage to 3Dpol (Fig. 2A), and it is these 14 isolates (Table 3) that account for the low but observable frequency of recombination between variants showing VP1 pairwise distances of <0.05 (6% to 8% of pairwise comparisons in the distance categories of <0.005, 0.005 to 0.1, and 0.1 to 0.25).

All sporadic E30 isolates involve unique ($n = 12$) or rare ($n = 1$) 3Dpol groups not found in any study or published E30 isolates characterized to date nor in any other species B enterovirus serotype (based on a GenBank search in May 2008). We have, however, detected one Spanish RF (ES27/Bb/Z/02) that falls into a VP1 lineage populated by RF-R variants but in the nonstructural region is a member of the large Z 3Dpol group. In this case, it must represent recombination between two E30 populations; the cocirculation of these RFs in 2002 in Europe (Fig. 4) is consistent with this possibility.

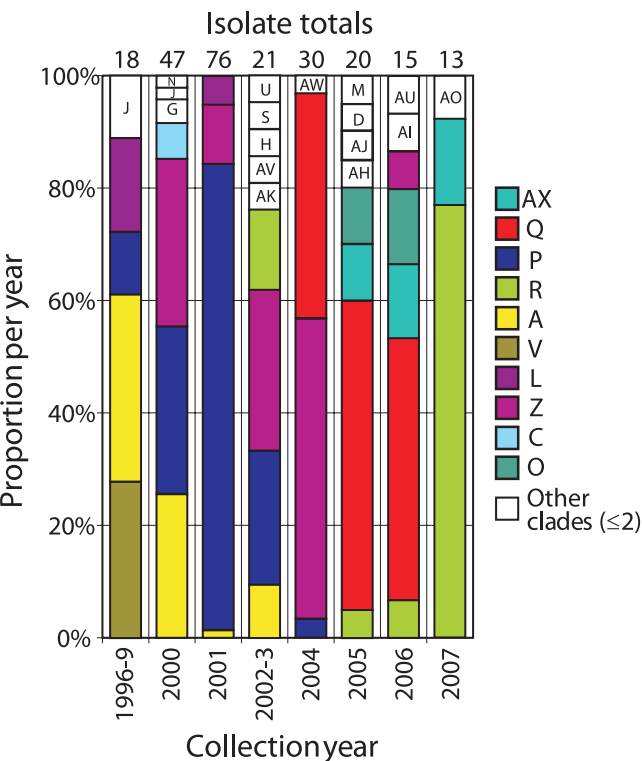


FIG. 4. Frequencies of different RFs in Europe over the observation period (240 isolates), color coded as for phylogenetic trees (see the legend of Fig. 1). RFs represented by two or fewer occurrences have been individually labeled in unfilled boxes.

DISCUSSION

Evolution of VP1 and 3Dpol regions. The data set analyzed in the current study was generated from one of the largest and certainly the most geographically diverse collections of enterovirus isolates assembled to date. Its study design allowed for

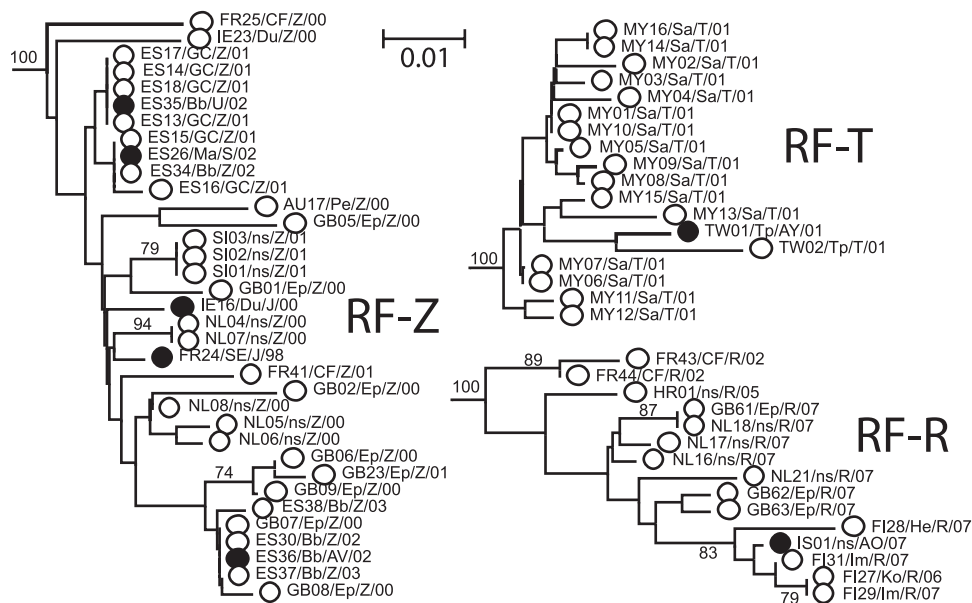


FIG. 5. Parts of VP1 phylogenetic tree showing lineages containing sporadic RFs (filled circles) with different 3Dpol groups from others in the clade (RF-Z, RF-T, and RF-R in the examples shown; isolates shown as unfilled circles). Trees are sections from a complete phylogenetic tree of all 318 study isolates constructed by neighbor joining using Jukes-Cantor corrected pairwise distances. The scale bar depicts an evolutionary distance of 0.1 used for the three trees shown and the complete tree of all isolates (not shown). Bootstrap resampling was used to determine robustness of the groupings; values of $\geq 70\%$ are shown.

the first time direct comparisons of E30 populations circulating worldwide, their transmission networks, and the temporal and geographical correlates of recombination. The collection of isolates assembled for the study fell predominantly in the genotype IIB lineage, as classified previously (42), consistent with the previously observed replacement of genotype I by genotype II E30 variants in the 1980s to 1990s (37, 42, 49). However, six

isolates from this study (two from Europe [GB60 and FI30] and four from Malaysia [MY18, MY19, MY22, and MY23]) collected between 2004 and 2007 fell into genotype I, demonstrating the continued, albeit reduced, circulation of these more divergent isolates until the present. Within the IIB group, variants fell into several previously recognized groups including the recently described subgroup H (8).

Sequence diversity in the VP1 region occurred predominantly at synonymous sites, consistent with previous analyses of variability of VP1 (4) and the whole P1 region (51). As discussed previously (4, 37, 49), the highly conservative nature of the substitutions that have accumulated in the diversification of E30 genotype II over the past 30 to 40 years does not immediately suggest that sequence change in structural gene regions is primarily driven by immune-mediated selection for antigenic change. The high rate of sequence change of the VP1 region characterized in this study (around 8×10^{-3} substitutions per site per year) (Table 2) lies within the range of substitution rates of other picornaviruses; among enteroviruses, rates of 13.5×10^{-3} for EV71 (species A), 9×10^{-3} for poliovirus serotype 1 (species C), and 5×10^{-3} for EV70 (species D) are described (7, 24, 55), similar to foot-and-mouth disease viruses (e.g., 4×10^{-3} to 14×10^{-3}) (reference 15 and references therein).

The pattern of sequence diversity in the nonstructural region (as exemplified by the phylogeny and sets of pairwise distances in the 3Dpol region analyzed in the current study) differed substantially from VP1. Differences include the existence of discrete clusters rather than the continuum of sequence distances in VP1 (Fig. 2) and the interspersed pattern of 3Dpol sequence groups of E30 with those of other species B serotypes that contrasts markedly with the monophyletic grouping of E30

TABLE 3. Characteristics of sporadic RFs

Variant ^a	Isolate source		3Dpol clade characteristics			
			Original ^b		New ^c	
	Year	Location	RF	Size (no. of isolates)	RF	Size (no. of isolates)
RU11	2000	Moscow	A	26	AN	1
ES8	2000	Madrid	A	26	N	1
HR02	2002	Croatia	L	4	AJ	1
ES63	2005	Gran Canary	Q	30	M	1
ES58	2005	Barcelona	Q	30	D	1
FI26	2006	Imatra	Q	30	AU	1
IS01	2007	Reykjavik	R	15	AO	1
MY17	2001	Sarawak	T	17	X	1
TW1	2001	Taipei	T	17	AY	1
ES26	2002	Madrid	Z	50	S	1
ES35	2002	Bilbao	Z	50	U	1
ES36	2002	Bilbao	Z	50	AV	1
FR24	1998	Southeast	Z	50	J	3
IE16	2000	Dublin	Z	50	J	3
ES27	2002	Bilbao	R	15	Z	50

^a RU, Russia; ES, Spain; FI, Finland; IS, Iceland; MY, Malaysia; TW, Taiwan; FR, France; IE, Ireland; HR, Croatia.
^b Identity and size of the 3Dpol clade associated with the lineage within which the sporadic isolate groups in VP1.
^c Designation and size of 3Dpol group of the sporadic isolate.

sequences in VP1 (including the more divergent genotype I sequences) (37, 42). These observations are consistent with the occurrence of frequent recombination and separate "modular" evolution of structural and nonstructural gene regions in species B and other enteroviruses (2, 13, 16, 20, 28, 29, 32, 35, 39–41, 47, 48, 51, 53). Our analysis of a large and geographically diverse set of E30 isolates in the current study provides evidence for an extremely large number of distinct 3Dpol lineages associated with E30 over the past 10 years and beyond; the finding that none was shared with other species B serotypes hints at the enormous diversity of these viruses circulating worldwide at any one time. What remains unclear is the time depth behind the current diversity of 3Dpol lineages and, by extension, this circulating pool of nonstructural region sequences with which E30 recombines. Regression analysis in the 3Dpol region analyzed (Table 2) failed to find a temporal relationship between the sample date and the sequence divergence from the tree root (in contrast to similar analysis of VP1), preventing calculation by BEAST of substitution rates in this region, or a date for the MRCA of the species B nonstructural region population. The latter date is important in further understanding timescales and sources of diversity in this region. It will also perhaps enable a dating of the timescale for the genesis of more than 40 different serotypes of this species since they seem biologically restricted to this nonstructural region population as recombination partners. In the future we propose to analyze much longer sequences in the P3 region on a wider range of sequences in each 3Dpol group to achieve this.

Recombinant forms of E30. In the current study we have used the 3Dpol group as a marker to identify different circulating populations of E30 recombinant forms. The data we have obtained add to previous investigations of E30 population turnover and geographical distributions based on the identification of E30 subpopulations by phylogenetic groupings in VP1 (1, 3, 4, 8, 37, 49, 59). The use of RF- and VP1-based classifications of E30 for these epidemiological investigations has its own advantages and disadvantages. Assignment of E30 into a series of RFs for population studies has the benefit of being unambiguous: recombination events are essentially irreversible, the assignment of 3Dpol sequences into different groups is relatively straightforward (e.g., by pairwise distances) (Fig. 2B), and there is no conceivable process by which 3Dpol sequences might resemble each other through evolutionary convergence except by multiple recombination events. In contrast, E30 classifications based on VP1 sequences have to impose divisions into what, ultimately, is a continuum of variability (Fig. 2A), reflected in part by the wide variety of different classifications with different resolutions of VP1 lineages that have been proposed to date (4, 37, 42, 49). On the other hand, the evolutionary process underlying the differentiation of VP1 can be more easily understood than possibly random or evolutionarily selected recombination events that lead to the appearance of new RFs; VP1 phylogenies represent a much better approximation of the evolutionary history of the E30 serotype than 3Dpol groups and allow time of origin and differentiation of structural gene regions to be more easily inferred. For these reasons, we advocate the use of both methods for investigation of the molecular epidemiology of E30 and other enteroviruses.

Our finding of a 3- to 5-year turnover and replacement cycle for individual RFs within Europe (Fig. 5) is consistent with previous VP1-based (3, 4, 8, 27, 37, 49) and combined VP1/RF-based (27, 32, 53) investigations of E30 population change within individual countries, such as Spain, France, the United Kingdom, and the United States. In one of the earliest studies (37), successive emergence and extinction of VP1 groups 1 to 4 was observed between 1956 and 1998 although in all likelihood (based on the diversity within each group and the time spans involved) they must each have contained a number of separate RFs. A more recent Spanish study identified emergence and extinction of five more closely similar VP1 lineages (all falling within genotype IIB, lineage F) over a period of 8 years (1992 to 2000), very similar to the pattern, shown in Fig. 4, which was observed throughout Europe in the subsequent 7 years. The use of similar time-correlated recombination analysis to that presented in Fig. 3 to a range of other species B serotypes found in blood from donors in the United Kingdom indicates that the rapid turnover observed for E30 is a general attribute of viruses within this species (53). This commonality, indeed, suggests the intriguing possibility of interserotype exchange of nonstructural gene region sequences as a source of 3Dpol diversity we have documented (see next section).

E30 transmission networks. The availability of samples from several European countries, Western Asia, Southeast Asia, and Australia allowed a detailed analysis of the geographical extent of the spread of individual RFs. Although numbers of samples from some areas were restricted, our comparative study provided evidence for a substantial commonality of E30 strains circulating at any one time in Europe, and additionally, at least in the year sampled (2000), in Australia. In contrast, all variants from Southeast Asia were entirely distinct throughout the study period. In terms of their geographical extents, these observations are consistent with previous observations of large-scale emergence and turnover of E30 variants identified as separate lineages in VP1 in samples from the United States (37) and Europe (49) and with detection of related viruses in isolates from widely separated countries. These include China and Japan (1), Western Russia and surrounding countries with Far Eastern Russia and Taiwanese variants (27, 61), and perhaps Spain and Argentina (42). Despite the intervening geographical bloc of Asia, which harbors different E30 populations from those circulating in Europe, the similarity in RF distributions between Australia and Europe in 2000 provides further evidence for very long-distance, rapid transmission of enteroviruses, in this case most likely through intercontinental air travel. These findings heighten concern about the potential rapid global spread of highly pathogenic enteroviruses that might emerge in the future, such as hemorrhagic conjunctivitis EV70 and neurovirulent EV71.

Despite the strong linkage between VP1 lineage (or sequence similarity) and 3Dpol grouping (Fig. 3A), a surprising new finding from the study was the frequent detection of sporadic recombinants of E30. These were embedded within VP1 lineages of E30 variants with a different 3Dpol group, such as the occurrence of an RF-AO isolate from Iceland deeply nested within a VP1 lineage of other European RF-R isolates. Of the 240 isolates analyzed from Europe, a total of 11 were sporadic, and 9 were found uniquely (Table 3); each of these was reamplified and resequenced to verify the presence of

these unexpected, discrepant 3Dpol groupings. Their frequent occurrence among circulating strains in both Europe and Southeast Asia (e.g., RF-T) suggests that there is continuing ongoing recombination during even extremely short-term evolution of E30, but in these instances, the variants did not expand sufficiently to replace the virus population (i.e., VP1 lineage) from which they originated. This hypothesis is supported by the pattern of replacement of the larger RF populations in which a new recombinant frequently emerges from within a VP1 clade of the RF it replaces. For example, the RF-R variants that first appeared in Europe in 2002 and continued to circulate until 2007 are direct evolutionary descendants of the older RF-P population (2000 to 2001). Similar emergence of RF-Z from within RF-L is also observed.

The underlying biological or epidemiological factors that govern which recombinants might be short lived or sporadic and which ones emerge and replace their ancestors are entirely unknown. As discussed previously (4, 49), it is difficult to imagine that the turnover of E30 variants results from a process of antigenic shift and drift that drive the evolution of structural proteins of influenza A virus. Although not formally tested, the low level of amino acid sequence variability in the VP1 region and other capsid proteins of E30 genotype II variants would be most unlikely to alter neutralization susceptibility and would therefore not allow a new RF to reinfect previously exposed and immune hosts. Likewise, the primary determinants of virus tropism are the receptor-binding capsid sequences, predicting that RFs probably exhibit a similar cell and tissue tropism. More difficult to discount is the possibility that successful RFs might have enhanced transmissibility, perhaps through achieving longer or more intense virus shedding after infection or greater infectivity on exposure. As discussed (21, 49), genetic determinants for such fitness differences may locate to the nonstructural region and thus vary between RFs. Finally, the process of population emergence, replacement, and extinction might be simply stochastic. Seasonality in E30 incidence alongside temporal and geographical differences in the proportion of human population susceptible to E30 infection potentially combine to create population bottlenecks that determine the chance survival and extinction of competing E30 populations year on year. As with other viruses that show unexplained rapid, large-scale genotype replacements, such as parvovirus B19 genotype 2 displaced by genotype 1 in the 1960s throughout the Northern hemisphere (36), our real understanding of virus population structures, transmission dynamics, host susceptibility changes, and possible evolutionary selection on the virus is currently so poor that these alternative explanations cannot be resolved.

Sources of viral diversity. A final unresolved issue is the immediate source of the wide range of 3Dpol lineages in our collection of E30 isolates. Emerging clearly from the finding of frequent sporadic RFs, the number of 3Dpol groups is much larger than the number of separately identifiable VP1 lineages, suggesting a source outside the E30 population. Although one isolate (ES27) showed evidence for recombination between two European cocirculating lineages (RF-R and RF-Z) (Table 3), it was remarkable that none of the others matched those of earlier E30 isolates circulating in the 1980s or 1990s or any other species B serotype characterized to date. However, this may be because there has been much less large-scale investigation of other commonly isolated enterovirus serotypes, such

as echovirus 9 and 11, CAV-9, and some of the coxsackie B viruses that have circulated contemporaneously with E30 over the past 10 years. The available data set for these viruses does not rule out the possibility of frequent interserotype recombination with E30, leading to extensive sharing of 3Dpol lineages with similar timescales for emergence and replacement of the major European 3Dpol lineages (A, P, Z, Q, and R). We are currently investigating this possibility through a second-phase collaborative investigation of 3Dpol sequence comparisons of E9 and E11 isolates with those of E30 over the same observation period of 2000 to 2007. From this, we may understand more about the genetic and epidemiological interrelationships of species B enteroviruses and the nature of their possibly interlinked evolution.

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